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Dvl3 translocates IPMK to the cell membrane in response to Wnt

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ABSTRACT

Wnt3a binds Frizzled-1 and the LRP5/6 co-receptors, ultimately activating Lef/Tcf-sensitive gene transcription in development. Inositol polyphosphate multikinase, IPMK, which possesses inositol phosphate kinase and lipid inositol kinase activities, is essential in Wnt3a regulation of its canonical pathway as well as physiologically in AMPK signaling. In the current report we show that translocation of IPMK to the cell membrane, where its substrates exist in high abundance, is obligate to its function in Wnt signaling. Translocation of IPMK to the cell membrane occurs within 5 min after Wnt3a stimulation. IPMK ducking onto Dishevelled-3 (Dvl3) requires a PDZ domain and the COOH-terminal prolyly-rich tail of Dvl3. Wnt3a-stimulates mobilization of Dvl3 to the cell membrane, translocating IPMK to the cell membrane also, to facilitate downstream signaling of Frizzled1. Deletion mutant of IPMK lacking the NH2-terminal variable region, IPMKΔN, fails to translocate to the cell membrane and to propagate canonical signaling. Targeting the IPMKΔN back to the cell membrane by addition of an isoprenylated CAAX box rescues its function in Wnt3a downstream signaling.

1. Introduction

Wnt activation of Lef/Tcf-sensitive transcription is the hallmark of the canonical pathway in development [1–3]. In mouse embryonal teratocarcinoma totipotent cells (F9), expression of rat Frizzled-1 confers Wnt3a sensitivity, activation of Lef/Tcf-sensitive transcription [4,5], and formation of parietal endoderm [4]. Inositol 1,4,5-trisphosphate 3-kinase (IP_3 3-kinase) and inositol polyphosphate multikinase (IPMK) are essential to production of inositol pentakisphosphate (IP_5) [6–9] and to canonical signaling by Wnt3a [10]. Previously we showed that Wnt3a stimulation of the canonical pathway generates IP_5, which acts to inhibit the activity of glycogen synthase kinase-3β, and to activate casein kinase 2. The consequence is accumulation of cytosolic β-catenin and subsequent activation of lymphoid enhancer factor/T-cell factor protein (Lef/Tcf)-dependent transcription [10]. Equally essential is the role of Dishevelled (Dvl), in particular Dvl3, in organizing large supermolecular complexes (i.e., signalsomes) necessary for signaling downstream from Frizzled-1 [11,12]. Dvl supermolecular complexes operate as mobile scaffolds, intracellular "toolboxes" that organize signaling partners temporally and spatially. To further understand the role of Dvl3 in IPMK signaling we interrogated the interaction between the Dvl3-based scaffold and this lipid kinase. Wnt3a is shown to stimulate docking of IPMK to the scaffold. Docking of IPMK to Dvl3 requires both the PDZ domain and COOH-terminal prolyl-rich tail of Dvl3. The elimination of either the DIX or the DEP domains does not affect docking of IPMK. The translocation of IPMK to the cell membrane rich in its substrate molecules requires both Dvl3 as well as Wnt3a activation.

2. Material and methods

2.1. Reagents

Recombinant mouse Wnt3a (Cat#1324-WN) was purchased from R&D System (Minneapolis, MN). Anti-Myc (M4439) and anti-β-catenin (C2206) antibodies were purchased from Sigma. Anti-Dvl3 (4D3, sc-8027) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor® 488 labeled secondary antibodies were obtained from Life Technologies (Grand Island, NY). The antibody against mouse IPMK was a kind gift from Dr. Sangwon F. Kim (University of Pennsylvania, School of Medicine, Philadelphia, PA). Small interfering RNAs were purchased both from Ambion (Corvallis, OR) and Life Technologies (Grand Island, NY).

2.2. Constructs

Rat IPMK sequence was amplified by PCR, digested by restriction enzymes *EcoRI* and *BglII* and inserted into peGFP-C1 vector (Clontech). A similar strategy was used to generate pcDNA3-based constructs of human IPMK with either Myc-tag or HA-tag at N-terminus of IPMK. For construction of hIPMKΔN (deletion of 1–77 amino acids from the N-terminus of human IPMK) the following primers were designed and employed to amplify the insert: 5′-GAAGATCTCTCAACCACCTCCAA GGGGC-3′ (forward primer); 5′-GGGGTACCTCAATTGTCTAAAATACTTC GAAGTACA-3′ (reverse primer). For construction of hIPMKΔN-CAAX (hIPMKΔN with a CAAX box at its C-terminus), the following primer

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pairs were used in PCR amplification: 5′-GAAGATCTCTCAACCACCTCCA AGGGGC-3′ (forward primer); 5′-GGGGTACCTTACATAATTACACACTTT GTCTTTGACTTCTTTTTCTTCTTTTTACCATCATTGTCTAAAATACTTCGAAGT ACA-3′ (reverse primer). Human Dvl3 cDNA fragments were generated by PCR amplification by using the following primers:

DIX domain, forward 5′-GGAATTCGGATGGGCGAGACCAAGATC ATC-3′, reverse 5′-CCGCTCGAGTCACTCAGCTGACACCAGCCA-3′; Linker 1 domain, forward 5′-GGAATTCGGGCCTCACACCCAGACCC A-3′, reverse 5′-CCGCTCGAGTCAGTTGAGTGACATGGTGGAGT-3′; PDZ domain, forward 5′-GGAATTCGGATCATCACGGTCACTCTCAA-3′, reverse 5′-CCGCTCGAGTCAGGCAACAGTCAGGGTGATGG-3′; Linker 2 domain, forward 5′-GGAATTCGGGCCAAGTGCTGGGACCC A-3′, reverse 5′-CCGCTCGAGTCACATGTCACTGTGGATGGACAA-3′; DEP domain, forward 5′-GGAATTCGGGCTGCCATCGTAAAAGCCA TG-3′, reverse 5′-CCGCTCGAGTCAGTCACCGAAGATGTAGTAGC-3′; Cter domain, forward 5′-GGAATTCGGCTCTGCGGCAACATGGCCAA C-3′, reverse 5′-CCGCTCGAGTCACATCACATCCACAAAGAA-3′.

PCR products were digested with *EcoRI* and *XhoI* and inserted into pGEX-4T-2 vector. All constructs were verified by DNA sequencing.

2.3. Cell culture and transfection

Mouse teratocarcinoma F9 cells were propagated in Dulbecco's modified Eagle's medium (Cellgro, Manassas, VA) supplemented with fetal bovine serum (15%, Hyclone, South Logan, UT), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C. All clones, simply termed the "clones" herein, were stably co-transfected with pcDNA3.1 harboring rat Frizzled-1 (Rfz1) and Lef/Tcf-luciferase reporter (Super8xTOPFlash, a kind gift from Dr. Randall Moon, University of Washington, Seattle, WA) were selected in complete medium containing neomycin analogue, G418 (0.4 mg/ml). At least three independent clones which contained both Rfz1 and Super8xTOPFlash were propagated. Lipofectamine 2000 and Lipofectamine RNAiMax (Life Technologies) were used to introduce plasmid DNA and siRNA, respectively, into cultured cells according to the manufacturer's instructions.

2.4. Immunoprecipitation and immunoblotting

Cells were harvested and lysed in lysis buffer containing 137 mM NaCl, 20 mM Tris (pH 7.5), 100 mM NaCl, 10 mM sodium molybdate, 1 mM Na_3VO_4, 10% glycerol, 1% NP-40 and a cocktail of protease inhibitors from Roche. Pellets were spun down by centrifugation at 14,000×*g* for 10 min at 4 °C. The supernatant (total cell lysates) was collected and protein concentration was measured as described. For pull-downs, 1 or 2 mg (protein) of total cell lysates were incubated with either anti-c-Myc agarose beads (#A7470, Sigma) or anti-Dvl3 antibody (Santa Cruz biotechnology) chemically linked to protein A/G agarose. Immune complexes were collected by centrifugation and washed with cold RIPA buffer (20 mM Tris, pH 8.0; 150 mM NaCl, 5 mM EDTA and 1% Triton X-100) for 3 times. Proteins collected by immune complexes were resolved from antibody-protein A/G agarose by boiling the sample in 20 μl of Laemmli solution for 5 min. Samples from immunoprecipitation or total cell lysates (50–100 μg protein/lane) were subjected to SDS-PAGE for separation. The resolved proteins were transferred onto nitrocellulose membranes. Blots were rinsed with buffer, incubated with primary and then secondary antibodies. Immune complexes were made visible by using an enhanced chemiluminescence method.

2.5. Knock-down protocol by using small interfering RNA

Duplexed siRNA with following sequences: UCUCCAGUGGUUU GAAAAUCAGAAG and CUUCUGAUUUUCAAACCACUGGAGA; GGAA GAGAUCUCGGACGACtt and GUCGUCCGAGAUCUCUUCCtt were used to target mouse IPMK and Dvl3 in F9 cells, respectively. To knock-down endogenous IPMK or Dvl3, reverse transfection was performed by using Lipofectamine RNAiMax, according to the manufacturer's protocol. Briefly, siRNA duplexes were mixed with Lipofectamine RNAiMax. The mixture was added into cell culture plates at a final concentration of 20 nM for siRNA duplexes. Suspensions of F9 cells were then seeded onto culture wells and cultured for 48 h. For rescue experiments, constructs of hIPMK were introduced into cells by using Lipofectamine 2000 at 24 h following addition of the siRNA targeting IPMK.

2.6. Lef/Tcf-sensitive transcription assay

F9 clones stably transfected with Rfz1 and Super8xTOPFlash were cultured in 12 well plates and stimulated with Wnt3a (20 ng/ml) for 5 h. Cell lysates were collected in reporter lysis buffer (Cat# E397A, Promega, Madison, WI). Cell lysates (10 μl) were incubated for 10 s with 100 μl of a reaction mixture containing 0.67 mM luciferin, 0.27 mM Coenzyme A, 0.1 mM EDTA, 1.1 mM $MgCO_3$, 4 mM $MgSO_4$ and 20 mM Tricine, pH 7.8. The intensity of luminescence was measured immediately using a luminometer (Lumat LB 9507, Berthold Technologies, Oak Ridge, TN). Samples were assayed in triplicate and the luciferase activity was normalized based on protein concentration.

2.7. Preparation of subcellular fractionation

Cells propagated in P150 Petri dishes were treated with or without purified Wnt3a for the time periods indicated in the figure legends. Cells were washed with phosphate-buffered saline twice, and then suspended in ice-cold buffer A (10 mM Hepes, pH 7.0, 5 mM $MgCl_2$, 25 mM KCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Cells were disrupted by repeated passage of cell suspension through a 23-gauge needle. An equal volume of ice-cold buffer A containing 0.25 M sucrose was added immediately after the disruption. Nuclei and unbroken cells were removed by centrifugation at 500×*g* for 10 min. The supernatant was transferred to a fresh tube and EDTA was added to a final concentration of 10 mM. The mixture was subjected to centrifugation at 16,000×*g* for 15 min. The resultant pellets were washed once with buffer A containing of 0.25 M sucrose and then were resuspended in a RIPA buffer. This subcellular fractionation yields a cell membrane-enriched fraction, high in cell membrane markers. The supernatant is employed as the source of cytosol.

2.8. Live cell imaging

For live cell imaging, F9 cells stably expressing Rfz1 were transfected with pcDNA3 harboring rat IPMK-eGFP and seeded onto collagen-coated glass-bottom culture dishes (MatTeck Corporation, Ashland, MA) for 24 h. Cells were stimulated with Wnt3a and images were immediately recorded by using a Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope (Microscopy Imaging Center, Stony Brook University).

2.9. Indirect immunofluorescence

Cells grown on glass-bottom dishes were first washed twice with DPBS buffer containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 140 mM NaCl, 1.5 mM KH_2PO_4 and 8 mM NaH_2PO_4 and then fixed with 3.8% formaldehyde for 15 min at room temperature. Fixed cells were permeabilized and incubated in DPBS buffer containing 2% bovine serum albumin for 20 min. Cells then were incubated with anti-c-Myc antibody (1:1000) at 37 °C for 30 min, washed with DPBS buffer for 3 times and then incubated with Alexa Fluor® 488 labeled secondary antibody at 37 °C for 30 min. Fluorescence staining

of Myc-IPMK was examined and recorded by using Olympus FluoView™ FV1000 confocal microscope.

2.10. Statistical analysis

All experiments were conducted minimally in triplicate sampling. Data are expressed as the means ± S.E. from at least three separate experiments. Comparisons of data among groups were performed with one-way analysis of variance followed by the Newman–Keuls test. Statistical significance (p value of less than 0.01) is denoted with asterisks or the pound symbols.

3. Results

3.1. IPMK regulates the Wnt canonical pathway

Functional analysis of the role of IPMK was probed in mouse F9 totipotent cells expressing rat Frizzled-1, employing siRNA reagents that target mouse IPMK (Fig. 1). Under these conditions, IPMK (44 kDa—M_r) was suppressed ~80% by targeted siRNA. IPMK expression was unaffected by treatment of the cells with commercially-prepared "control" siRNA (Fig. 1A). Downstream activation of Lef/Tcf-sensitive transcriptional activation was analyzed using the Super8xTOPFlash reporter (Fig. 1B). At 5–7 h post Wnt3a treatment, Lef/Tcf-sensitive transcription was activated. Treating cells with siRNA targeting mouse IPMK sharply reduced Wnt3a activation of the canonical pathway. Lipid-based signals provoked from Wnt3a activation are derived, in part, by IPMK (as well as IP3K). Transient expression of Myc-tagged human IPMK (Myc-hIPMK) in the IPMK-deficient cells fully "rescues" the ability of Wnt3a to stimulate the canonical pathway. Treating cells with commercially-prepared "control" siRNA likewise did not alter the ability of Wnt3a to stimulate Lef/Tcf-sensitive transcription. By measuring changes in cytosolic β-catenin levels, Wnt3a signaling could be assessed at 1 h post-stimulation (Fig. 1C). Wnt3a stimulates increased accumulation of cytosolic β-catenin. Treating cells with siRNAs targeting mouse IPMK abolished the β-catenin accumulation in response to Wnt3a. Transient expression of human IPMK in the siRNA-treated cells "rescues" the ability of Wnt3a to elevate cytosolic β-catenin levels (Fig. 1C), confirming its ability to "rescue" Wnt3a-stimulated Lef/Tcf-sensitive transcriptional activation at 5 h post-stimulation (Fig. 1B).

3.2. Wnt3a stimulates IPMK translocation to the cell membrane

IPMK is localized largely in the cytosol and nuclei (Fig. 2A). Inositol trisphosphate (IP_3) and inositol tetrakisphosphate (IP_4), substrates for IPMK, are generated at the inner leaflet of the cell membrane via hydrolysis of phosphatidyl inositol bisphosphate by phospholypase Cβ [13] and subsequent phosphorylation of IP_3 by IP_3 3-kinase [14]. IPMK acts of these substrates to generate IP_5 in response to Wnt3a [10]. How IPMK translocates to the cell membrane in response to Wnt3a is a question central to understanding the operation of the canonical pathway. To answer this query, we investigated the effects of Wnt3a on IPMK translocation. IPMK tagged with eGFP was expressed in F9 cells and fluorescence microscopy performed to examine its localization (Fig. 2A). Treating the cells with Wnt3a provoked a rapid translocation of IPMK to the juxtamembrane and cell membrane regions. IPMK could be observed in both regions even in the absence of Wnt. Treating the cells with Wnt3a provoked more pronounced migration of IPMK to the cell membrane and

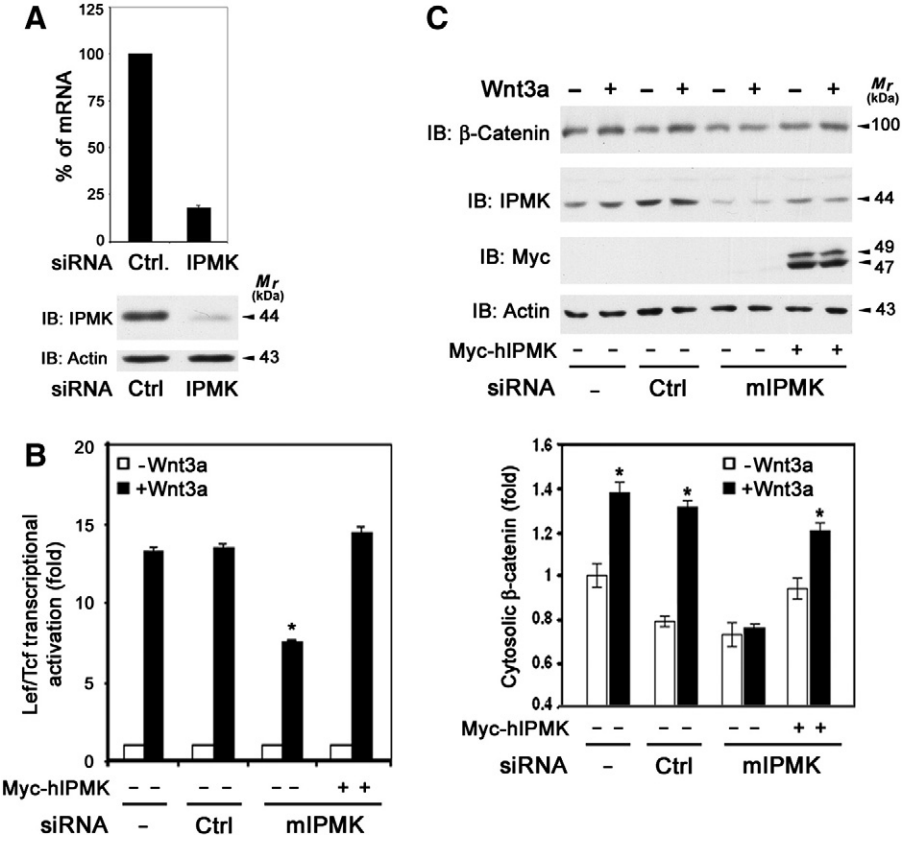

Fig. 1. IPMK regulates Wnt3a-stimulated canonical signaling. A, Knockdown of IPMK by siRNA in F9 cells was analyzed by qPCR and immunoblotting. Commercial-designed and synthesized oligos were employed as "control" for siRNA treatments (Ctrl). Immunoblots of actin displayed in panel A and C establish equivalent loading of protein samples. B and C, Clones were treated with vehicle (−), control siRNA (Ctrl) or siRNA targeting IPMK (mIPMK) for 24 h and thereafter cells were transfected with or without plasmids harboring Myc-tagged human IPMK (Myc-hIPMK). Twenty four hours later, cells were treated with (+) or without (−) Wnt3a for 1 h (C) or 6 h (B). Reporter activities (B) and cytosolic levels of beta-catenin (C) were analyzed and graphed. B *$p<0.001$ versus control (−siRNA, +Wnt3a) group; C *$p<0.01$ versus control (−siRNA, −Wnt3a) group.

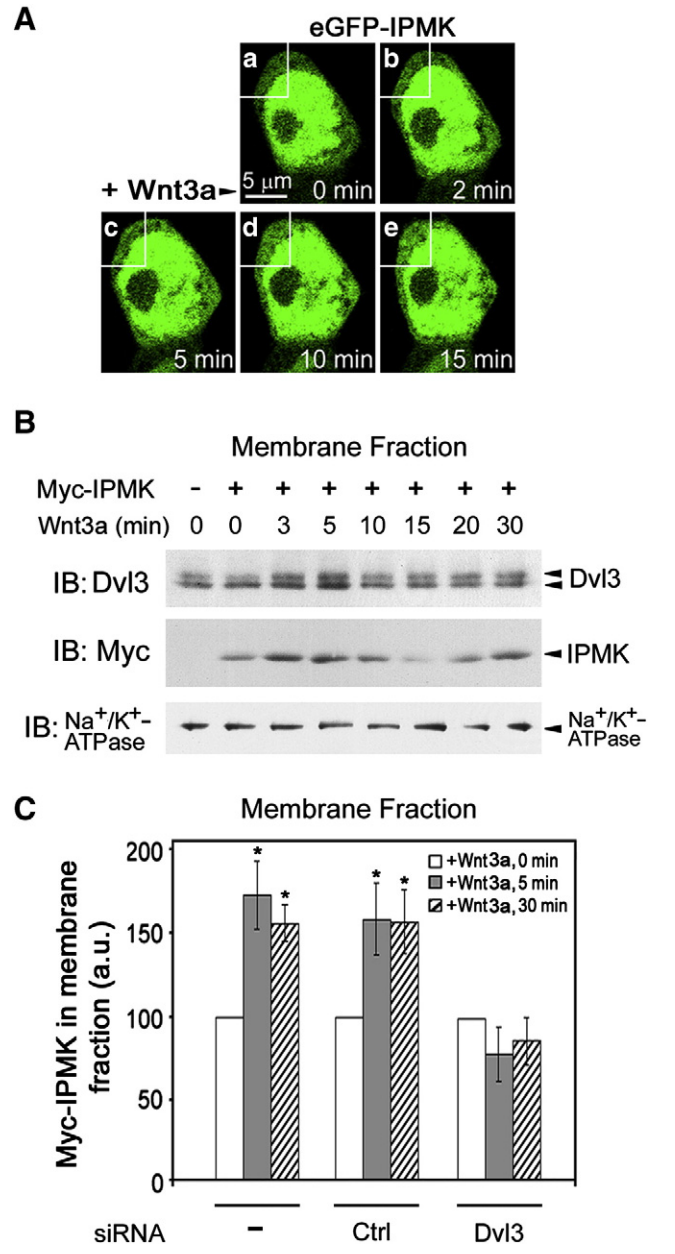

Fig. 2. Wnt3a stimulates IPMK translocation to the cell membrane. A, eGFP-tagged IPMK was expressed transiently in F9 clones. Cells were cultured on a glass-bottom culture dish and stimulated by Wnt3a. The fluorescence images of eGFP-IPMK in live cells were captured by using confocal microscopy as described in Materials and methods. B, F9 clones were transiently transfected with either empty vector (−) or pcDNA3 harboring Myc-IPMK for 24 h. Wnt3a was added into culture media and thereafter cells were harvested at the indicated time periods. Cell membrane-enriched subcellular fractions were isolated according to the protocol described. Dvl3 and Myc-IPMK content in cell membrane fractions were probed by immunoblotting. Immunoblots of Na^+/K^+-ATPase mark cell membrane-enriched fractions and demonstrate equal loading of samples. C, Clones were treated with vehicle (−), control siRNA (Ctrl) or siRNA targeting Dvl3 for 24 h. Cells then were transfected with a plasmids harboring Myc-IPMK for 24 h. Cell membrane-enriched subcellular fractions were collected from cells treated with Wnt3a for 0, 5 and 30 min, respectively. Dvl3 and Myc-IPMK in each sample were probed by immunoblotting and their relative abundances in samples were compared by intensities of immunoblotting bands corresponding to Dvl3 and Myc-IPMK. *$p<0.01$ versus control (−siRNA, +Wnt3a at 0 min) group.

juxtamembrane region. The increase in cell membrane localization of IPMK was obvious within 5 min of stimulation by Wnt3a. By 10–15 min post-stimulation with Wnt3a, accumulation of IPMK at the plasma membrane was prominent. These results observed by fluorescence microscopy were tested biochemically, by examining the amounts of Myc-tagged IPMK that can be found associated with the cell membrane. A cell membrane-enriched (Na^+/K^+-ATPase-enriched) subcellular fraction was probed for IPMK content (Fig. 2B, C). Both IPMK and Dvl3 association with the cell membrane was established in this subcellular fraction using immunoblotting (Fig. 2B). A time-dependent accumulation of Dvl3 in the cell membrane-enriched subcellular fraction in response to Wnt3a was observed. In addition, Wnt3a stimulated a rapid, transient increase in the amount of IPMK found in cell membrane-enriched fraction (Fig. 2B, C).

The possibility that Dvl3, a mobile scaffold which migrates to the cell membrane in response to Wnt3a, may be involved in IPMK targeting to the cell membrane was tested. If Dvl3 translocates IPMK, loss of Dvl3 should block the translocation. To test this hypothesis, we made use of siRNA to knock-down Dvl3 expression. We examined Myc-IPMK content in cell membrane-enriched fractions prepared from Dvl3-deficient F9 cells stimulated by Wnt3a at 0, 5 and 30 min. Knockdown of Dvl3 effectively abolished Wnt3a-stimulated translocation of IPMK to the cell membrane (Fig. 2C). In contrast, treating cells with control siRNA affected neither expression of Dvl3, nor translocation of IPMK to the cell membrane in response to Wnt3a (Fig. 2C).

3.3. Wnt3a stimulates IPMK docking to Dvl3

We probed if the translocation of IPMK in response to Wnt3a involved docking to Dvl3. Pull-downs of Dvl3 were prepared from lysates of F9 cells transfected with Myc-tagged IPMK or vector alone (Fig. 3A). Dvl3 is a phosphoprotein, herein displaying $M_r = 78$ and 80 kDa, reflecting the multiple phosphorylated nature of this scaffold protein [12,15]. Wnt3a treatment stimulated increased content of Myc-tagged IPMK in pull-downs of Dvl3 from lysates of F9 cells treated with Wnt3a over a 30-min time course (Fig. 3B). The relative amount of IPMK found in the Dvl3-based pull downs was maximal at 5 min post Wnt3a stimulation, reaching about 6–7-fold over basal, untreated conditions (Fig. 3C). This increase in Wnt3a stimulation in IPMK associated with Dvl3 was transient, falling from the maximum at 5 min to less than control levels by 30 min poststimulation. IPMK associates with Dvl3, which is observed in Dvl3-based, very large supermolecular signalsomes that have been observed and characterized by fluorescence microscopy (in vivo), large-bore steric exclusion chromatography (in vitro) and fluorescence correlation spectroscopy (in vivo, live cell, real-time measurements) (Wang and Malbon, unpublished data).

3.4. Docking of IPMK requires the PDZ domain and COOH-terminal tail of Dvl3

Dvl3 is a multivalent scaffold with several well-known domains (e.g., DIX, PDZ, DEP, and prolyly-rich C-terminal tail) that bind protein kinases (serine, threonine, and tyrosine-specific), phosphatases, and adaptor molecules [16]. We interrogated each of these domains of Dvl3 to assess if IPMK docking to Dvl3 was dependent on their individual integrity. Myc-tagged human Dvl3 and a Dvl3 deletion mutant (Fig. 4A) were co-expressed with HA-tagged IPMK in the F9 cells under standard conditions. Cells were lysed and pull-downs of Myc-tag were performed on the whole-cell lysates (Fig. 4B). The levels of expression for Myc-tagged Dvl3, or for Myc-Dvl3 mutants were similar among transfected cells. Likewise, HA-tagged IPMK expressed in these cells showed at similar levels (Fig. 4B, panels of total lysates). The pull-downs of Dvl3 (IP: Myc-tag) were probed for bound IPMK, which was HA-tagged. Results from immunoblotting of these pull-downs revealed IPMK in the complexes of Myc-tagged Dvl3. The Dvl3 mutants lacking the DIX (Δ-DIX) or DEP (Δ-DEP) domains actually showed a modest increased content of bound IPMK than observed for the wild-type Dvl3. In contrast, two of the mutants, Δ-PDZ and Δ-C-terminal tail Dvl3, failed to dock IPMK (Fig. 4B). Studies in vitro assaying IPMK binding to purified Dvl3 domains

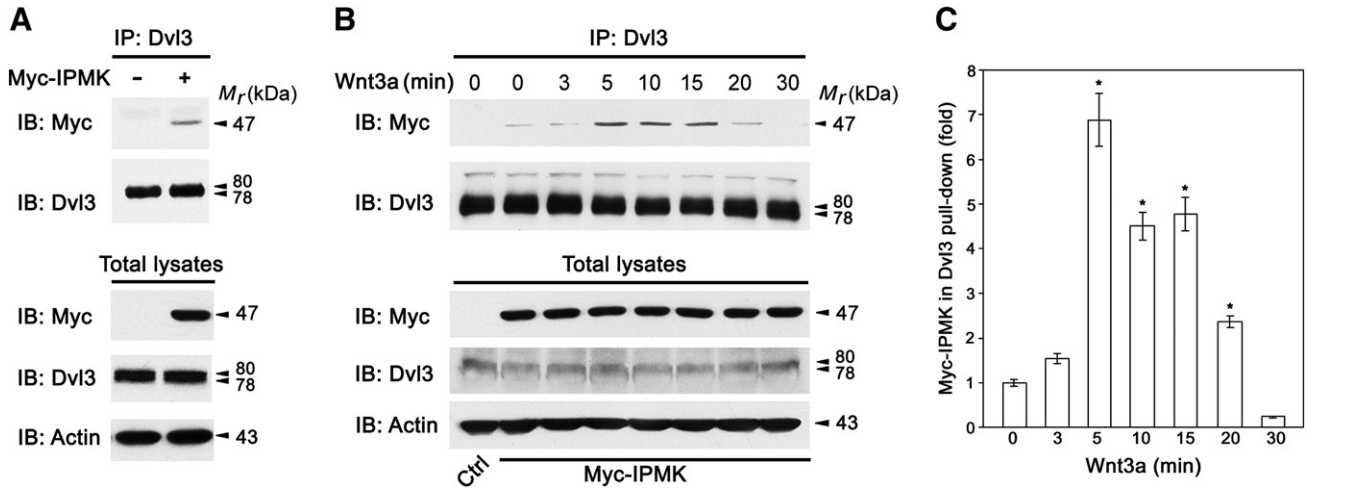

Fig. 3. IPMK docking to Dvl3 in response to Wnt3a. F9 clones were transfected with either an empty vector (− or Ctrl) or an expression vector harboring Myc-IPMK. A, Immunoblots of Myc-IPMK and Dvl3 from Dvl3 pull-downs (top panel) and from total cell lysates (bottom panel) were displayed. B, Clones were treated with Wnt3a and cell lysates were collected at indicated time periods. Immunoblots of Myc-IPMK and Dvl3 from Dvl3 pull-downs (top panel) and from total cell lysates (bottom panel) were prepared. At least 3 independent experiments were conducted for each experimental design. Immunoblots of actin are shown to demonstrate equal loading of protein samples. C, The intensities of Myc-IPMK in blots obtained from at least three sets of Dvl3 pull-downs were compared and plotted, setting the value at 0 min of Wnt3a treatment as 1. *$p<0.01$ versus control (Wnt3a at 0 min) group.

confirmed that both the PDZ domain as well as the C-terminal tail of Dvl3 bind purified IPMK (data not shown). IPMK docks to Dvl3 directly, in a manner which is dependent minimally upon the presence of a PDZ domain and the C-terminal tail of this scaffold protein.

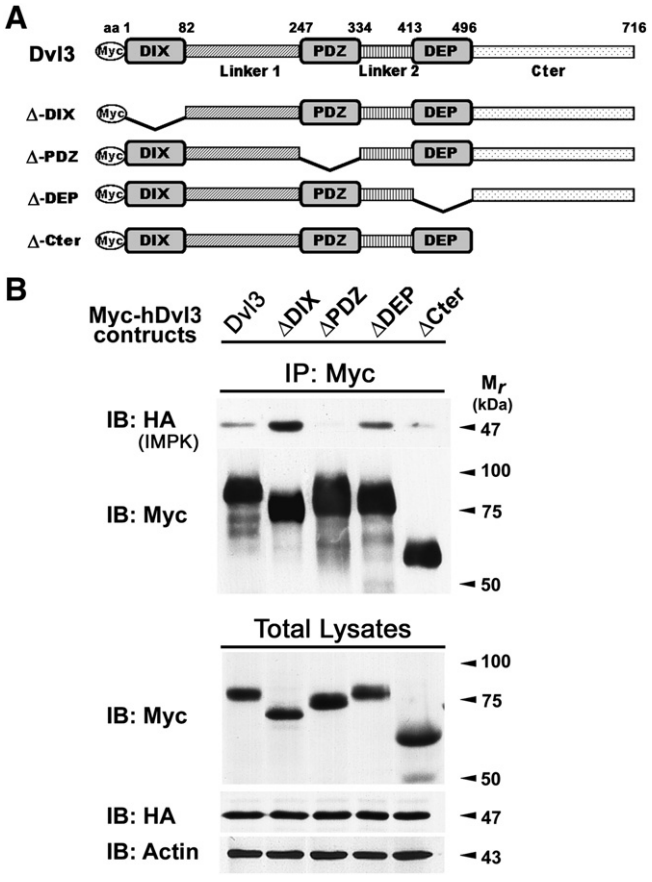

Fig. 4. Deletion of either the PDZ domain or the C-terminal "tail" of Dvl3 blocks IPMK docking. A, Schematic of Myc-Dvl3 and deletion mutants of Dvl3. B, One Myc-tagged Dvl3 deletion mutant and HA-tagged IPMK were co-expressed in F9 cells. Immunoblots of Myc and HA obtained from pull-downs by Myc-antibody (top panel) and total lysates (bottom panel) are displayed.

3.5. N-terminal region of IPMK contributes to membrane targeting

Having identified the PDZ domain and C-terminal tail of Dvl3 as necessary for IPMK docking, we sought to explore the basis of IPMK translocation towards plasma membrane in response to Wnt3a stimulation. All but the first 60 amino acids at the NH_2-terminus of mouse IPMK (mIPMK) are proposed as a kinase domain composed of structures involving with substrate binding and catalytic activity as it was reported for yeast IPMK [17]. We probed the role of the N-terminus of IPMK by deletion mutagenesis (Fig. 5A). We treated F9 cells with siRNA targeting expression of endogenous IPMK. Cellular IPMK content was suppressed by siRNA treatment (Fig. 5B). The functional status of IPMK mutants could best be interrogated by "rescue" experiments, in which expression of a mutant form of human IPMK (hIPMK) were assessed for their ability to overcome the loss of endogenous IPMK. The siRNA targeting mouse IPMK reduced, but did not abolish, mIPMK expression in F9 cells. These IPMK-deficient cells were then transfected with expression vectors harboring hIPMK and assayed for function. The N-terminal deletion of 77 amino acids of hIPMK (hIPMKΔN) is the human equivalent to N-terminal deletion of 60 amino acids of a mouse homologue (Fig. 5A). The loss of the N-terminal region of hIPMK resulted in sharply diminished IPMK content of the cell membrane-enriched subcellular fraction (Fig. 5C), which is enriched in Na^+/K^+ ATPase.

Since N-terminal region is proven to be important for IPMK translocation, the second mutant tested the hypothesis that targeting the hIPMKΔN to the cell membrane artificially would overcome interruption in translocation of hIPMKΔN. The approach makes use of introducing a "CAAX-box" to the C-terminus of a protein. The presence of a CAAX-box ensures prenylation of the Cys residue, the proteolytic cleavage of the three C-terminal amino acids, and methylation of the isoprenylcysteine residue, which acts as an independent lipid anchor to the cell membrane [18]. The hIPMKΔN mutant was engineered to accommodate a C-terminal CAAX-box (hIPMKΔN-CAAX), ensuring that the mutant lacking the Dvl3 targeting sequence in the N-terminus would still localize to the cell membrane. Fluorescence microscopy indeed localized the expressed hIPMK-CAAX mutant largely to the cell membrane (Fig. 5D). Cells expressing hIPMK as well as mutant forms of hIPMK were assayed for their ability to respond to stimulation by Wnt3a. The read-out was the activation of the Lef/Tcf-sensitive transcriptional pathway, the hallmark of the canonical Wnt pathway (Fig 5E). Deficiency of endogenous mIPMK sharply attenuated the ability of Wnt3a to stimulate the activation of Lef/Tcf-sensitive

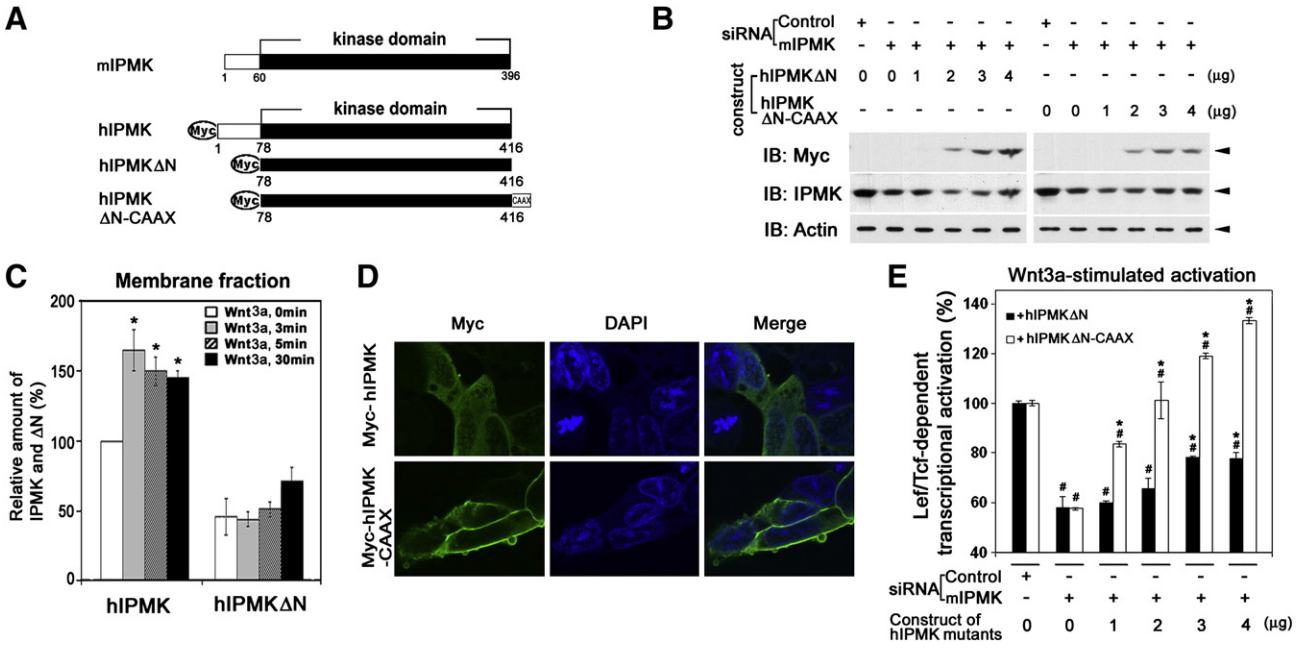

Fig. 5. N-terminal region of IPMK targets the cell membrane in response to Wnt3a. A, Schematic of IPMK, ΔNhIPMK, and ΔNhIPMK-CAAX. B, F9 clones were treated with either control siRNA or siRNA targeting IPMK for 24 h. Expression vectors harboring either Myc-ΔNhIPMK or Myc-ΔNhIPMK-CAAX were introduced into siRNA-treated mIPMK-deficient cells, using an increasing amount of plasmid DNA, as indicated. Immunoblots of Myc, IPMK and actin (as a loading control) are displayed. C, Cell membrane-enriched fractions from cells expressing either hIPMK or ΔNhIPMK were collected from cells that were challenged with Wnt3a for the time period indicated. IPMK and ΔNhIPMK content in cell membrane-enriched subcellular fractions were probed by immunoblotting. The content of each of these proteins in the blots was analyzed by scanning densitometry. The relative amounts of each protein found are graphed. *$p<0.01$ versus control (hIPMK, Wnt3a at 0 min) group. D. Indirect immunofluorescence images of F9 cells expressing either Myc-ΔNhIPMK or Myc-ΔNhIPMK-CAAX. DAPI was used to stain nuclei. E, F9 clones were treated with either a "control" siRNA or siRNA that targets mouse IPMK. Increasing amounts of expression vectors harboring either Myc-ΔNhIPMK or Myc-ΔNhIPMK-CAAX were employed to transfect the siRNA-treated mIPMK-deficient cells. Twenty four hours after transfection, the cells were stimulated by Wnt3a for 6 h. Cell lysates were collected and transcriptional reporter Luciferase activity used to measure Lef/Tcf-dependent transcriptional activation. The Wnt3a-stimulated activation revealed by the reported read-out is displayed. The reporter activity established for the cells treated with "control" siRNA-treated group was set as 100%. #$p<0.01$, versus control (control siRNA treated) group; *$p<0.01$ versus knockdown (mIPMK siRNA-treated clones with and without hIPMK or mutant expressed) group.

transcription. Expression of hIPMK to mimic normal endogenous levels fully restored Lef/Tcf-sensitive transcription in response to Wnt3a. Expression of the hIPMKΔN mutant had only a limited ability to rescue Lef/Tcf-sensitive transcription. This inability to fully rescue provides a functional consequence for the earlier noted inability of the N-terminal deletion mutant to associate with the cell membrane, even when cells were stimulated with Wnt3a (Fig. 5C). Expression of a hIPMKΔN-CAAX mutant that is targeted to the cell membrane by the presence of a C-terminal isoprenylcysteinyl residue not only "rescues" Wnt3a-stimulated activation of Lef/Tcf-sensitive transcription, but surpasses it by more than double (Fig. 5E).

4. Discussion

Dishevelleds function as mobile multivalent scaffolds [19]. As such, Dvls organize the functions of a myriad of proteins that dock to Dvl3-based signalsomes to function in Wnt signaling, as well as perhaps other signaling pathways. Proteins docking to Dvl3-based scaffolds include protein kinases, phosphoprotein phosphatases, Frizzled G-protein-coupled receptors, members of the β-catenin destruction complex, and others [16]. In the current work the repertoire of docking proteins for Dvl3 is expanded to include IPMK, a protein kinase implicated in the inositol metabolism. The observations reinforce the role of IPMK in Wnt signaling [10]. Most notable is that suppression of IPMK expression with siRNA attenuates the ability of Wnt to activate canonical signaling.

An association of IPMK with Dvl3 is shown at several levels. The docking of IPMK to Dvl3 was established by pull-downs of Dvl3 that revealed the presence of IPMK. Further, the amount of IPMK associated with Dvl3-based supermolecular complexes increased in response to treating the cells with Wnt3a. Wnt3a stimulates increased association of IPMK with the cell membrane. Based upon the ability of Wnt3a to provoke translocation of Dvl3 to the cell membrane [20], we speculated that in response to Wnt3a, IPMK docking to Dvl3 and migration of IPMK to the cell membrane might be linked. As metabolism of inositol-containing lipids and their derivatives of water-soluble inositides occur in the proximity of the cell membrane, migration of IPMK via docking to Dvl3-based scaffold in response to Wnt3a might provide a mechanism for integrating the water-soluble inositides and downstream Wnt signaling. Docking studies in vitro also demonstrate that IPMK binds to Dvl3 (data not shown). The binding is direct and requires PDZ and C-terminal domains of Dvl3 for IPMK docking. This observation does not preclude, however, the possibility that IPMK might dock to additional proteins other than Dvls during Wnt-stimulated signaling.

More insightful were results of studies of two mutant forms of IPMK that focused on the leading N-terminal region of the kinase. The first mutant lacked the N-terminal 1–77 amino acid region of the native IPMK, hIPMKΔN. The region is unstructured and not necessary for kinase function. Expression of the hIPMKΔN mutant allowed us to probe if the N-terminus of the IPMK was involved in cell membrane targeting. In cells made deficient in endogenous IPMK, expression of the hIPMKΔN mutant failed to rescue Wnt signaling. The reduction in function likely reflects the inability of the IPMKΔN mutant to associate with the cell membrane in vivo, in response to Wnt3a. We tested whether the loss-of-function observed for the ΔN mutant of IPMK could be reversed if the protein could be directed to the cell membrane by an independent mean. To artificially target hIPMKΔN to the cell membrane, a CAAX-box was added to the C-terminus. The resultant hIPMKΔN mutant lacking the N-terminal (1–77) region localized to the cell membrane. More importantly, hIPMKΔN-CAAX expression was able to rescue the loss-of-function induced by the ΔN mutation in the context of IPMK-deficient cells. Not only did the hIPMKΔN-CAAX

rescue the function, it dramatically potentiated the effects of Wnt3a on Lef/Tcf-dependent transcriptional activation.

IPMK catalyzed IP_5 formation and is an important signaling molecule in Wnt canonical signaling [10]. IPMK activity and translocation to the cell membrane is necessary for Wnt activation of the canonical signaling. Like other key proteins in these signaling pathways, IPMK docks to the Dvl3-based scaffold. IPMK can dock Dvl3 directly, although this does not preclude IPMK docking to other proteins in the very large Dvl3-based signalsome. The unique need for a mobile scaffold like Dvl3 to organize docking molecules at the cell membrane is reinforced herein by studies of IPMK. The dynamic character of Dvl-based signalsome formation and translocation to the cell membrane is well documented, established biochemically by steric-exclusion chromatography on large-bore matrices, optically, by fluorescence correlation spectroscopy in live cells challenged with Wnt3a, and by simple fluorescence microscopy that first revealed very large Dvl-based punctae behaving as we now know signalsome do [11,12,21]. Each of these distinctly different methodologies of interrogation discern Dvl3-based complexes to be large, dynamic in character, functioning and changing locale in response to Wnt signaling. IPMK plays a critical functional role and the current work now places IPMK within the context of Dvl3-based signalsome that translocates to the cell membrane early in the activation of the canonical pathway by Wnt.

5. Conclusion

IMPK mediates Wnt3a activated canonical signaling pathway. Translocation of IPMK to plasma membrane via docking Dvl3 is important for IPMK to access substrates and generate IP_5, activating down-stream signaling.

Disclosure of potential conflicts of interest

No potential conflicts of interest was disclosed.

Authors' contributions

Y.W. performed experiments. Y.W. and H.Y.W. designed the research plan, analyzed data and wrote the manuscript.

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